Reference: *Biol. Bull.*, 137: 265–276. (October, 1969)

STUDIES ON A TREHALASE FROM A SYMBIONT OF THE TROPICAL COCKROACH, *BLABERUS CRANIIFER*— A SUGGESTED ANALYTICAL ENZYME [1]

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Herein we report the characterization of a trehalase (α,α-1-glucoside 1-glucohydrolase, EC 3.2.1.28) isolated from a bacterial symbiont from the midgut of *Blaberus craniifer*, a tropical cockroach.

Although this is not the first isolation of such a hydrolase, the present study will disclose that this source of enzyme has one advantage over previously reported sources, *i.e.*, crude preparations of this enzyme do not contain large amounts of any other carbohydrases which would release glucose from glucose-containing oligosaccharides or polysaccharides and thus interfere with the qualitative or quantitative estimation of trehalose in complex biological fluids or extracts. The absence of other hydrolases simplifies purification procedures and insures the accurate quantitative estimation of trehalose in mixtures containing such oligosaccharides and polysaccharides.

Materials and Methods

Culture conditions

The microorganism, a gram negative bacillus, apparently a species of *Aerobacter*, was obtained from the midgut of *Blaberus* and isolated by routine plating techniques on minimal salt media where trehalose served as the only carbon source. The isolated strain was grown in minimal salt medium (Heath and Ghalambos, 1962) plus 5% trehalose at 34° in a rotary incubator for 24–36 hours. The cells were removed by centrifugation at 46,000 *g* for 30 minutes at 2° and washed by further centrifugation in the minimal salt medium without carbohydrate.

Isolation and purification of the trehalase

The washed cells were take up in distilled water (1:3 w/v) and stored at −20° overnight. The cell suspension was thawed and refrozen three times and subjected to intermittant sonication for six minutes at 0°. Cell debris was removed by centrifugation at 50,000 *g* at 0° for 20 minutes and the clear supernatant adjusted to 0.05 *M* with $MnCl_2$. This preparation was maintained at 0° for 30 minutes and cleared by centrifugation at 30,000 *g* at −1°. The resultant supernatant was brought to 30% (v/v) with −20° reagent grade acetone and the mixture held at −7° for five minutes before centrifugation at 30,000 *g* at −10°. The acetone supernatant was discarded and the precipitate suspended in 50 ml 0.001 *M* citrate

[1] This research was supported by grant C-239 from the Robert A. Welch Foundation.
[2] Holder of NIH Pre-Doctoral Fellowship #GM 37711.

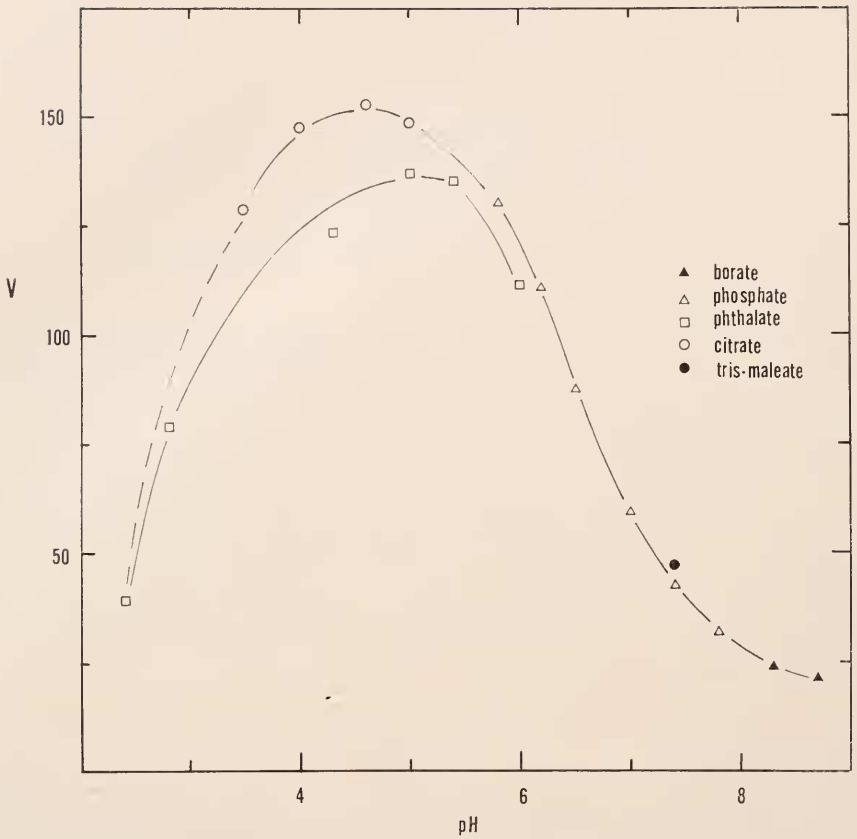

FIGURE 1. Effect of pH on trehalase activity; incubation mixtures contained in a final volume of 0.325 ml: 10 μmoles trehalose, 50 μmoles buffer and 0.025 ml enzyme.

buffer at pH 5.0. After gentle stirring at 0° for 30 minutes the undissolved material was removed by centrifugation at 46,000 × *g* at 0° for 30 minutes. The acetone fraction was then subjected to column chromatography at 3° on DEAE cellulose prepared according to the method of Peterson and Sober (1956). The 10 ml column was equilibrated with 0.005 *M* phosphate buffer pH 7.4 and stepwise elution of the enzyme was effected by the addition of 30 mls of each the following buffers at 0.005 *M*: phosphate pH 7.4 and 6.5 followed by citrate at pH 5.8 and 4.6. Following elution with the above buffers, a second elution of 0.005 *M* citrate buffer at pH 4.6 containing increasing amounts of NaCl was conducted in the following sequence: 0.01 *M*, 0.1 *M*, 0.5 *M* and 1.0 *M*. The eluted enzyme was dialyzed at 4° C against 0.005 *M* citrate buffer at pH 4.6 and the dialysate reduced to a powder by lypholyzation.

Assay of enzyme and analytical methods

The standard assay system contained 50 μmoles of buffer, 10 μmoles of substrate and 0.025 ml of enzyme in a total volume of 0.325 ml. Deviations from this

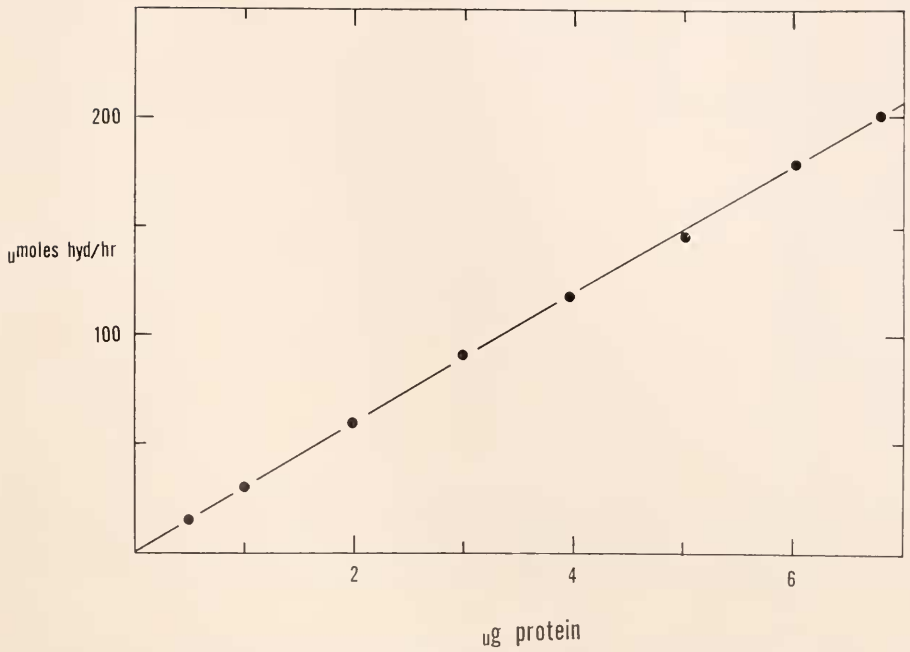

FIGURE 2. Effect of enzyme concentration on rate of trehalose hydrolysis; incubation mixtures contained in a final volume of 0.325 ml: 10 μmoles substrate, 50 μmoles citrate buffer at pH 4.6 and 0.025 ml enzyme.

assay system will be discussed in the text. Reaction mixtures were incubated for 15 minutes at 30°. To estimate the products formed in the assay system two techniques were employed: (1) Glucose-oxidase (Sols and de la Fuente, 1957) (β-D-Glucose: oxygen oxidoreductase, EC 1.1.3.4): o-dianisidine ("Glucostat," Worthington Biochemical Corp.) made up in 0.2 *M* Tris: HCl buffer, pH 7.2 (Fisher, 1964) was added to the reaction mixture, substrate blanks and glucose standards and incubated for 10 minutes at 30°. This reaction was stopped and the color developed by the addition of 0.05 ml of 8.0 *N* HCl. The tubes were read in a Coleman Universal spectrophotometer at 410 mμ or in a Klett-Summerson colorimeter with a #42 filter. (2) Alternatively the incubation mixture was subjected to the alkaline copper method of Somogyi (1952) for detecting reducing sugars. In addition some estimations were made exposing the reaction mixture to hydrolysis in 0.2 *N* NaOH at 100° for 20 minutes which degraded the enzymatically liberated glucose and the remaining trehalose was estimated by the phenol-H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). In cases where protein interfered with the above estimations, the reaction was subjected to equal volumes of $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1952). After clarification by centrifugation aliquotes of the supernatant were assayed by the glucose oxidase and/or the alkaline-copper method. Protein was estimated by the Folin-Ciocalteu reagent (Lowry, Rosenbrough, Farr and Randall, 1951) using crystalline bovine serum albumin as the standard. Enzymatic activity is expressed as μmoles substrate hydrolyzed/mg protein hr^{-1}.

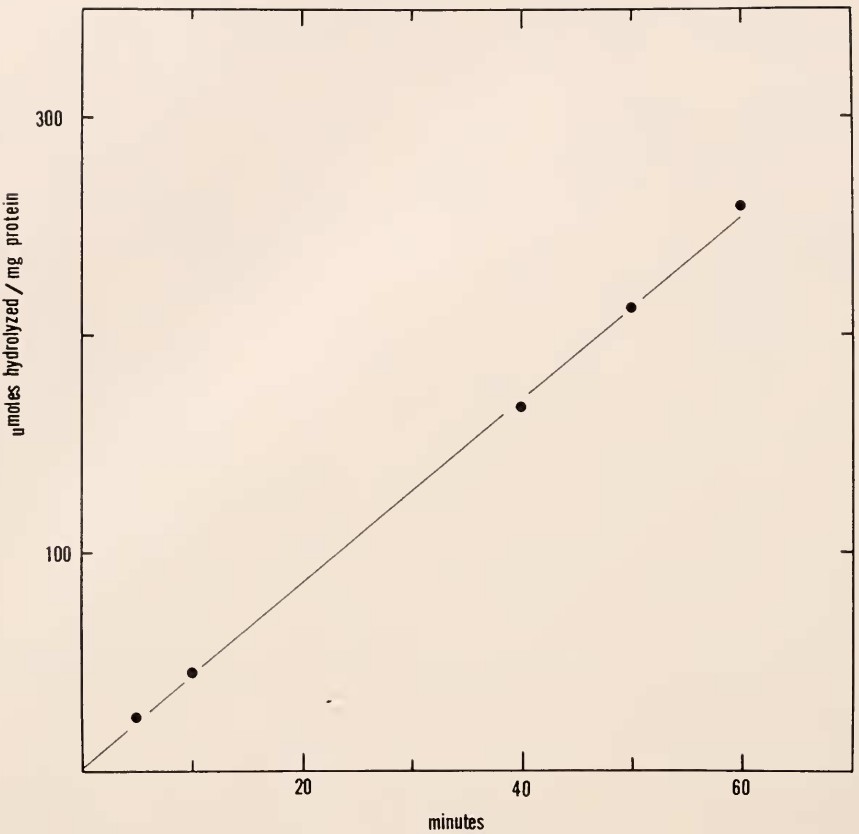

FIGURE 3. Effect of time on rate of trehalose hydrolysis; incubation mixtures contained in a final volume of 0.325 ml: 20 μmoles trehalose, 50 μmoles citrate buffer at pH 4.6 and 0.01 ml enzyme.

Chemicals

All chemicals used in these experiments were reagent grade. Substrates were purchased from Pfanstiehl Chemical Company and/or Sigma Chemical Company.

RESULTS

Effect of pH

Figure 1 illustrates the effect of pH on the rate of hydrolysis of trehalose. Data presented in that figure is based on the average of four determinations at each pH. The optimal pH in citrate buffer was found to be about 4.6. Phthalate buffers at the same concentration and covering the same pH range caused about 13% inhibition of enzymatic hydrolysis. No inhibition of hydrolysis was observed when Tris (hydroxymethyl) amino methane: maleate buffers were examined.

TABLE I

*Effect of metals on enzymatic activity**

Addition	Sp Act	% Inh
—	149.6	0
$CaCl_2$	136.8	8.6
$NiCl_2$	134.4	10.2
$CdCl_2$	119.2	20.4
$ZnCl_2$	110.4	26.2
$CoCl_2$	94.4	36.9
—	149.0	0
CsCl	100.0	32.9
LiCl	99.2	33.5
NaCl	96.0	35.6
KCl	96.0	35.6
RbCl	94.4	36.4
PCMB	147.0	0

* Incubation mixtures contained in a final volume of 0.525 ml: 50 μmoles citrate buffer, pH 4.6, 10 μmoles substrate and 0.025 ml enzyme. Divalent inhibitors were used at 10 μmoles, monovalent metals at 200 μmoles and PCMB at 0.033 μmoles. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose-oxidase with inhibitor controls.

Effect of time, characterization of the product, effect of enzyme concentration and substrate concentration

Figure 2 indicates that enzymatic hydrolysis of trehalose was linear with respect to enzyme concentration over the range examined 0.05 to 7 μgm) and is based on triplicate observations. The product of hydrolysis was identified as glucose in four chromatographic solvents: butan-1-ol, pyridine, water (6:4:3); propan-1-ol, ethyl acetate, water (7:1:2); butan-1-ol, ethanol acetone, water (5:4:3:2); ethyl acetate, pyridine, 2-butanone, water (50:36:36:30). Following development of the chromatograms with alkaline silver nitrate (Trevelyan, Procter and Harrison 1950), only two spots were visualized, corresponding to glucose and trehalose. Eluted glucose areas were further examined by the alkaline copper reagent for total reducing sugar, glucose oxidase specific for glucose and total carbohydrate by the phenol:H_2SO_4 method. Results from these reactions gave the following ratios: 1.04:1.0:1.02.

Enzymatic hydrolysis was also linear with respect to time (Fig. 3) when examined over a 60 minute period. The rate of hydrolysis of trehalose was examined over a 100 fold range using triplicate tubes at each concentration (Fig. 4) and the K_m was estimated to be 2.24×10^{-3} *M*. The V_{max} was calculated to be 289.9 μmoles hydrolyzed/mg protein hr^{-1} from the double reciprocal plot (Figure 4) derived from the average of four determinations. Maltose, cellobiose, α-methyl glucoside and glucose at 0.05 *M* and starch and glycogen at 0.1 mg/ml did not influence the K_m or V_{max} indicated above.

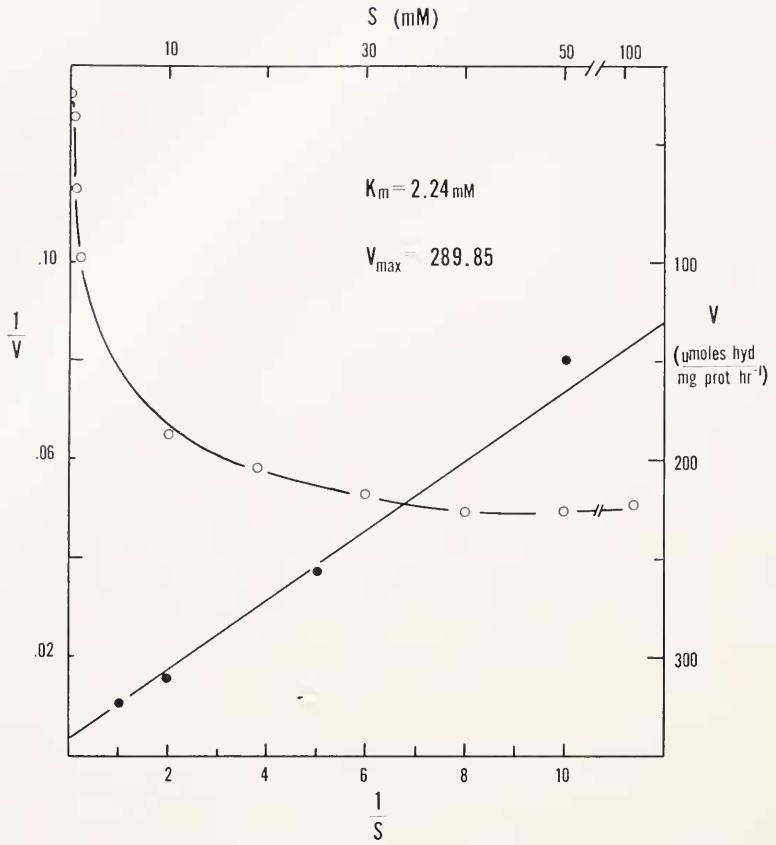

FIGURE 4. Effect of substrate concentration on trehalase activity; incubation mixtures contained in a final volume of 1.225 ml: 100 μmoles citrate buffer at pH 4.6, 0.025 ml enzyme and trehalose in various amounts.

Effect of inhibitors

The effect of metal ions on the hydrolysis of trehalose is presented in Table I. Maximum activity is based the average of three estimations with citrate buffer at pH 4.6. At relatively low concentrations the divalent metals inhibited the enzyme from about 9% to 37%. At much higher concentrations the monovalent metals inhibited the trehalose about 35%. No effect was noted when PCMB was used at 10^{-5} *M*.

Purification of trehalase on DEAE cellulose and enzyme specificity

Figure 5 presents the elution profile form the DEAE cellulose for protein, hydrolytic activity as well as hydrogen ion and Cl^- concentration of each fraction.

The stages of the purification presented in the materials and methods section are presented in Table II. With this procedure the enzyme was purified about 80 fold. Examination of the purified enzyme indicated that the pH optimum was

the same as that in the crude preparation. Table III presents the specificity of the crude and the purified fraction against various glucose containing substrates indicates that no other carbohydrates were detected in the crude fraction and that no other enzymes were further purified on the DEAE column aside from trehalase.

TABLE II

*Enzyme purification**

Fraction	Volume (ml)	Protein (mg)	Sp. act	Units act	Purification
Crude	70.0	724.5	7.30	5288.0	0
Mn^{++} super	73.5	437.2	14.82	7082.0	2.03
Mn^{++} ppt	70.0	280.4	0.10	28.0	—
30% acet ppt	70.0	259.3	27.00	7001.1	3.70
50% acet ppt	35.0	20.9	0.11	2.3	—
Acet ppt super	65.0	44.7	146.5	6548.6	20.06
DEAE	15.0	38.4	488.0	22,579.2	80.54

* Incubation mixtures in a final volume of 0.325 ml contained: 50 μmoles citrate buffer, pH 4.6, 10 μmoles trehalose and 0.025 ml enzyme. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose oxidase with substrate controls. The alkaline-copper reagent was used in parallel assays.

TABLE III

*Substrate specificity of trehalase**

		μmoles Hydrolyzed mgm protein hour	
		Crude	Purified
Trehalose	α 1 → 1	7.3	588.0
Kojiobiose	α 1 → 2	0	0
Maltose	α 1 → 4	0.1	0
Isomaltose	α 1 → 6	0	0
Sophorose	β 1 → 2	0	0
Laminaribiose	β 1 → 3	0	0
Cellobiose	β 1 → 4	0	0
Gentiobiose	β 1 → 6	0	0
Sucrose	α 1 → 2	0.8	0
Melibiose	α 1 → 6	0	0
Lactose	β 1 → 4	0	0
Melezitose	α 1 → 3, β 2 → 1	0.1	0
Raffinose	α 1 → 6, α 1 → 2	0	0
α-Methyl-glycoside	α → 1	0	0
Starch	α 1 → 4	0	0
Glycogen	α 1 → 4	0	0

* Incubation mixtures in a final volume of 0.325 ml contained: 50 μmoles citrate buffer, pH 4.6, 10 μmoles substrate, and 0.025 ml enzyme. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose-oxidase with substrate controls.

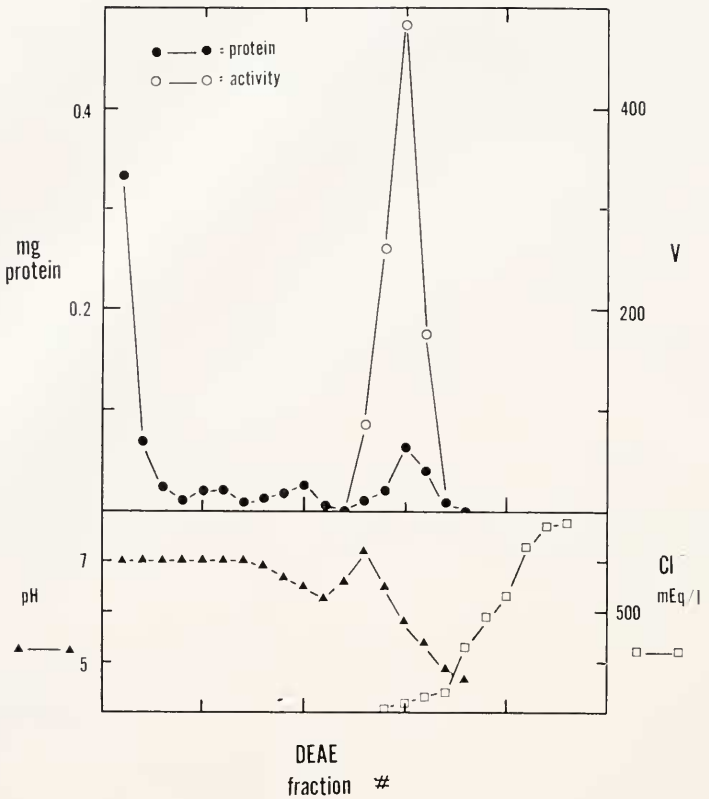

FIGURE 5. Purification of trehalase on DEAE cellulose column. Details of elution are presented in the text.

Energy of activation

The energy of activation of trehalose hydrolysis was determined from the linear portion (0° to 60°) of an Arrhenius plot (log K *vs* 1/T) where the slope is equivalent to -E/2.303R (Fig. 6) and found to be 9321 Cal/mole from duplicate experiments. It was also observed that 86% of the enzymatic activity remained after one hour at 70° providing substrate was present, whereas 40% of the hydrolytic activity was lost in 10 minutes at 70° in the absence of trehalose.

DISCUSSION

During the past ten years considerable interest has been directed toward the study of trehalases from divers sources. A resume of these investigations is presented in Table IV. With the identification of trehalose as the predominant circulating sugar in insects (Wyatt and Kalf, 1957) many investigations have been directed toward the trehalases from this class of arthropods. The further identification of trehalose in a number of invertebrates with representatives from 12 phyla (Fairbairn, 1958) and in a number of protists (Cochrane, 1961; Sussman,

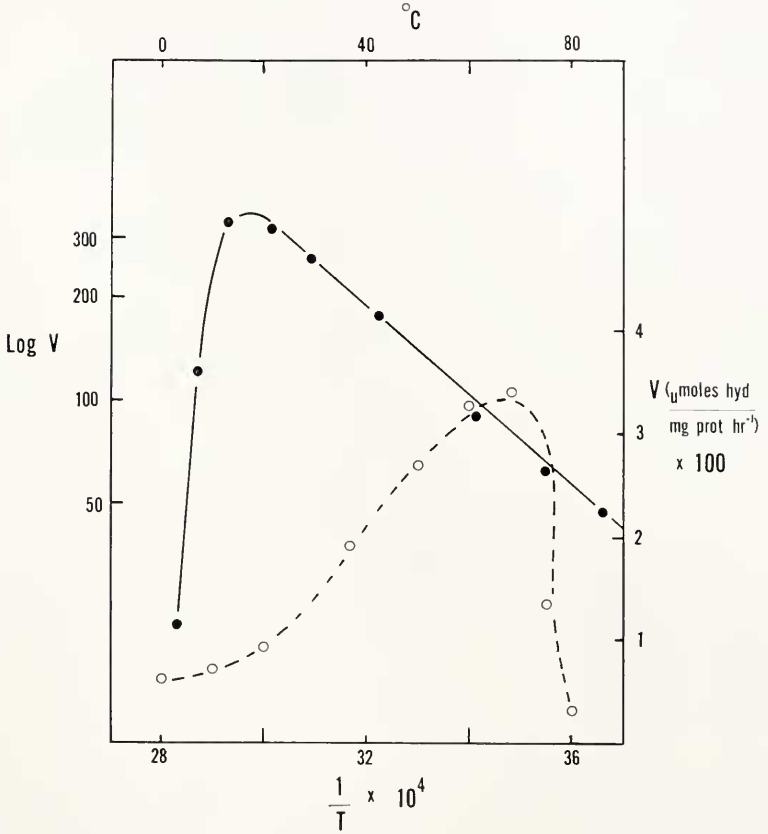

FIGURE 6. Arrhenius Plot (1/T *vs.* log K) of trehalase activity; incubation mixtures contained in a final volume of 0.325 ml: 50 μmoles citrate buffer pH 4.6, 20 μmoles trehalose and 0.025 ml enzyme.

1961; Clegg and Filosa, 1961) has increased the interest in this sugar. It is of considerable importance therefore to have a rapid accurate means of assaying this non-reducing disaccharide. We feel the trehalase isolated from the symbiont of *Blaberus* represents a source for such an analytical enzyme.

The trehalase reported herein has a pH optimum of 4.6 which is somewhat lower than most of the enzymes heretofore reported, one exception being that of the trehalase from *Aspergillus oryzae* reported to have optimum activity at pH 4.0 (Horikoshi and Ikeda, 1966). Courtois, Petek and Kolahi-Zanovzi (1962), also reported that an enzyme from yeast possessed two optima, and at 5.3 and one at 4.1, however, the data presented by those authors did not reveal well defined peaks of hydrolytic activity at either the above hydrogen ion concentrations. The pH optimum and K_m of the trehalase from the symbiont of *Blaberus craniifer* is quite similar to that reported by Gilby, Wyatt and Wyatt (1966) for the midgut trehalase of *Blaberus discoidalis*. Those authors obtained a 100,000 *g* supernatant fraction from homogenates of washed gut tissue. Whether there was any relation-

TABLE IV

Characteristics of some reported trehalases

Source	pH optimum	Km (m*M*)	Author
Aspergillus oryzae	4.0	2.5	Horikoshi and Ikeda, 1966
Symbiont from *Blaberus craniifer*	4.6	2.2	Fisher and McAlister, present paper
Blaberus discoidalis, midgut	5.0	0.5	Gilby *et al.*, 1966
Bombyx mori, whole pupae	5.2	0.44–.47	Satio, 1966
Yeast	5.3 (4.1)	0.9	Courtois *et al.*, 1962
Bombyx mori, larval midgut	5.4	2.9	Horie, 1959
Melanoplus differentialis, whole adults	5.5	5.1	Derr and Randall, 1966
Neurospora crassa	5.5	0.27 (pool)	Hill and Sussman, 1964
Galleria mellonella, whole larvae	5.5	0.13 sic	Kalf and Rieder, 1958
Dictyostelium discoideum	5.5	1.2	Ceccarini, 1966
Phormia regina, whole adults	5.6	0.67	Friedman, 1960
Saccharomyces cerevisiae	5.7	0.41	Panek and Souza, 1964
Hyalophora cecropia, larval midgut	5.7	0.40	Gussin and Wyatt, 1965
Phormia regina, flt. musc. mito.	5.8	1.3	Hansen, 1966
Blaberus discoidalis, thr. musc. micr.	6.0	3.3	Gilby *et al.*, 1966
Swine Intestine	6.0	3.0	Dahlquist, 1960
Hyalophora cecropia, thr. musc. micr.	6.5	3.6	Gussin and Wyatt, 1965
Streptomyces hygroscopius	6.5	18.0	Hey and Elbein, 1968
Plantago ovata	6.5	0.3	Courtois *et al.*, 1962
Melolontha vulgaris, whole adults	6.5	0.7	Courtois *et al.*, 1962
Hybrid yeast (#12836)	6.9	10.2	Avigad *et al.*, 1965

ship between a bacterial enzyme similar to that described in this paper and the gut trehalase described by Gilby *et al.* (1966) is unknown, but comparative studies on such enzymes would be of interest. Such speculation on the role of symbionts in the enzymology of insect gut has been proposed by Duspiva (1954). It is also of interest that the enzyme described in the present report does not show any inhibition by phosphate or Tris buffers. Such inhibition of trehalases by phosphate buffers has been reported by Hey and Elbein (1968) for the enzyme from *Streptomyces hygroscopius*. Inhibition of trehalases by Tris buffers have been reported by Hey and Elbein (1968), Gilby *et al.* (1966) and Friedman (1960), for enzymes from *S. hygroscopius*, *Blaberus discoidalis* and *Phormia regina*.

The Km for the symbiont enzyme is $2.2 \times 10^{-3} M$ which is markedly similar to most reported trehalases; however that enzyme characteristic is quite different from that reported by Hey and Elbein (1968) and Avigad, Ziv and Neufeld (1965) for the trehalases from *Streptomyces* and a hybrid yeast. The relatively low Km of the enzyme here described is quite advantageous when this enzyme is considered for the analysis of trehalose in biological materials since that sugar often occurs at very low concentrations (Fairbairn, 1958). The energy of activation of this trehalase is about 9300 Cal/mole which is consistant with a large number of carbohydrases, however, it is considerably lower than that reported for the trehalase from *Blaberus discoidalis* muscle microsomes as 15,000 Cal/mole by Gilby *et al.* (1966).

By simple acetone precipitation and passage of the present of enzyme through a DEAE cellulose column the enzyme was purified 80 fold and the specificity for trehalose was maintained. No hydrolysis was observed when 16 other glucose containing oligo- and polysaccharides were examined. The yield of enzyme obtained from symbionts grown on glucose, cellobiose and maltose did not differ significantly from those grown on trehalose; however, the yield of bacterial cells was much less when cellobiose and maltose was used as the only carbon source of interest in this line is the fact that cells grown on maltose and cellobiose did not have any enhanced α $1 \rightarrow 4$ or β $1 \rightarrow 4$ glucosidase activity.

The role of this symbiont in the physiology of *Blaberus* remains obscure. Wyatt (1967) proposed that the trehalase of the insect intestine would prevent the loss of trehalose via the feces since that disaccharide upon entry into the intestine by diffusion would be hydrolyzed to glucose within the gut and again pass into the hemocoel as glucose via diffusion. Again it is not known if the symbiont participates in this function. The trehalase of the symbiont is not an exoenzyme and it would appear that this point would rule out any effect on maintenance of trehalose levels within the insect.

Summary

1. A trehalase from a gram negative symbiont of *Blaberus craniifer* has been isolated and purified 80 fold.

2. Hydrolysis of trehalose was linear with respect to time and protein concentration.

3. The pH optimum of the enzyme was found to be 4.6, the K_m 2.2×10^{-3} M and the V_{max} about 290 μMole trehalose hydrolyzed/mgm protein hr^{-1}.

4. The energy of activation is found to be 9321 Cal/mole and enzymatic hydrolysis was inhibited by monovalent and divalent metal; however, no effect of the sulfhydryl inhibitor PCMB was noted.

5. The merit of this source and this trehalase as an analytic reagent is discussed.

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